IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of Grotendorst, *et al.*	Art Unit: 1812
	Examiner: L. Spector
Serial No.: 07/752,427 Filed: August 30, 1991	
FOR: **CONNECTIVE TISSUE GROWTH FACTOR**	

DECLARATION UNDER 37 C.F.R. §1.131

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

I, Gary R. Grotendorst, Ph.D., declare and state that:

1. I am a co-inventor of the subject matter described and claimed in the United States Patent Application Serial No. 07/752,427, filed on August 30, 1991, entitled, "Connective Tissue Growth Factor".

2. I am familiar with the prosecution history of Patent Application Serial No. 07/752,427.

3. I understand that the Examiner contends that the specification is objected to under 35 U.S.C. §112, first paragraph, as allegedly failing to adequately teach how to make and/or use the invention, i.e., failing to provide an enabling disclosure. Claims 14-16 are rejected under 35 U.S.C. §112, first paragraph, for the reasons set forth in the objection to the specification.

4. The specification is objected to as allegedly not providing enablement for the production of antibodies, either monoclonal or polyclonal, that react with the protein of the invention or fragments thereof, but do not cross-react with platelet derived growth factor (PDGF).

5. In contrast to the comments in the Office Action, the production of polyclonal and monoclonal antibodies is routine using the current techniques available. We have identified a unique sequence in CTGF which corresponds to amino acid residues 232-259 (See peptide GG105, Figure 1, Appendix A). We have produced goat antibodies to this specific sequence of CTGF. Similar antibodies have been produced that specifically recognize the PDGF sequence. Neither the CTGF specific antibodies nor the PDGF specific antibodies cross-react with the other protein.

Briefly, we utilized a routine experimental protocol for the production of polyclonal antibodies which specifically bind CTGF, but not PDGF. Peptide GG105, a 28 amino acid peptide (Figure 1, Appendix A) was used to immunize goats (50μg) in Freund's complete adjuvant by multiple intradermal injections. All subsequent immunizations were with 50 μg of peptide in Freunds incomplete adjuvant. Immune sera were collected seven days after the fourth challenge and subsequent challenges. Western blot analysis of the immune sera (Figure 2, Appendix B), showed that anti-CTGF antibody reacted well with CTGF but did not exhibit any cross-reactivity to PDGF. (Figure 2, Appendix B: Lane 1 shows molecular weight markers; Lane 2, COS7 cells transfected with the plasmid pcDNAI, expressing CTGF open reading frame in antisense orientation; Lane 3, COS7 cells transfected with pcDNAI, expressing CTGF open reading frame in sense orientation; Lane 4, Recombinant PDGF AB (10ng)). Panel A was probed with anti-PDGF IgG and Panel B

was probed with anti-CTGF (GG105 peptide) antiserum. The results clearly show that the anti-PDGF IgG detects both PDGF and CTGF, as seen in Panel A, lanes 3 and 4. In sharp contrast, the anti-CTGF antisera detected only the CTGF-peptide (Panel B, lane 3) and not the PDGF peptide (Panel B, lane 4), demonstrating the specificity of the antibody.

Multiple bands were detected in the recombinant protein western blot when analyzed with anti-PDGF under non-reducing conditions (Appendix B, Figure 2). These are all CTGF peptides since they are not present in the control or antisense samples. We believe that the variation is due to different degrees of glycoslylation. This is supported by the finding that the anti-GG105 antibody detects a single size class of peptide in the media after reduction with DTE. Because this sequence is near the middle of the CTGF peptide, we would detect smaller fragments if they were produced by internal cleavage of the protein by proteases. This is not the case. Thus, CTGF is a monomeric protein and is not composed of 18.5 kDa peptide fragments or 12 kDa peptide fragments as suggested in the Campochario and Shimakado manuscripts. No PDGF is detected by this antisera.

6. I understand that the Examiner has rejected claims 1 and 4 under 35 U.S.C. §102(b) as anticipated by or, in the alternative, under 35 U.S.C. §103 as obvious over Matsuoka, *et al.*, or alternatively Campochiaro, *et al.*, or alternatively Shimokado, *et al.*

Expression of CTGF gene in several cell types was tested. Total RNA was isolated from cultured cells by acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA (10 μg) was electrophoresed on an agarose/formaldehyde gel (1.5%) and transferred to nitrocellulose. The CTGF probe was a ^{32}P-

labeled 2.1 kb EcoRI insert of clone DB60R32 in the Bluescript Vector (Bradham, *et al.*, *J. Cell Biol.*, 114(6):1285, 1991) (Figure 3, Appendix C). RNA samples were isolated from: HUVE, human umbilical vein endothelial cells; CON-FIB, human foreskin fibroblasts (control); TGF-β-FIB, human foreskin fibroblasts (treated with 10 ng/ml of TGFβ for 4 hours); NEU, human peripheral neutrophil activated with F-met-leu-phe (100 ng/ml for 6 hours); MAC, human peripheral blood monocytes activated with LPS (10 μg/ml for 6 hours). The fibroblasts expressed low levels of the CTGF gene and expressed higher levels after activation with TGF-β. Neutrophils and macrophages did not express detectable CTGF gene.

7. To confirm that the cDNA for CTGF produced the same polypeptide of the present invention, recombinant CTGF was produced and analyzed by Western blot. The open reading frame of the CTGF mRNA was amplified using specific oligonucleotide primers that contained Eco R1 sites and this was cloned into the pcDNA I vector from Invitrogen (San Diego, CA) (Figure 4, Appendix D). Recombinant protein was collected from conditioned by COS 7 cells that were transfected with vector containing no insert (control) sense orientation of the CTGF ORF or antisense orientation of the CTGF ORF. The media was then analyzed by Western blot using anti-PDGF antibodies as in Figure 2A (Appendix B) and Figure 5 (Appendix E) or with anti-GG105 peptide (anti-CTGF) antibodies as in Figure 2B (Appendix B).

8. The biological activity of the media containing recombinant CTGF was tested with the COS cell media. Mitogenic assays were performed using 48 well plates and NIH 3T3 cells as target cells. The cells were plated in DMEM, 10% FCS and the 3T3 cells made quiescent by incubating for 2 days in serum-free DMEM containing ITS supplement (Collaborative Biomedical,

Bedford, MA) before use. Varying amounts of the conditioned media containing the recombinant CTGF and known amounts of recombinant PDGF standard were added to the wells and the plates incubated at 37°C in 10% CO_2, 90% air for 18 hours, after which ^{3}H-thymidine at a final concentration of 5 uC/ml was added and incubated for an additional 2 hours. The media was removed, the cells washed and DNA synthesis determined from the ^{3}H-thymidine incorporation into trichloroacetic acid precipitable material by scintillation counting.

As seen in Figure 6 (Appendix F), the recombinant CTGF stimulates a mitogenic response comparable to that of recombinant PDGF BB. NC is media from antisense transfected cells. There are approximately 15 ng/ml of CTGF in the conditioned media based on the amount of biological activity compared to PDGF. These studies show that the recombinant CTGF encodes the CTGF polypeptide which exhibits mitogenic activity.

9. As co-author on the Matsuoka, *et al.* reference (*Proc.Natl.Acad.Sci. USA*, 86:4416, 1989), I have recently reviewed the reference and it has become evident that there is a printer's error in the legend to FIGURE 4B. The error indicates that the mitogenic activity of the anti-PDGF immunoabsorbed fraction is indicated by the solid circles and the total wound fluid activity by the open circles. The opposite is correct, as is clearly shown by the discussion of the figure in the text of the reference. The legend for FIGURE 4C correctly states that the total chemotactic activity is indicated by the solid circles and the anti-PDGF immunopurified samples are the open circles. This makes the peak of anti-PDGF immunopurified mitogenic and chemotactic activities day 1, not day 4 or 5.

10. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date June 11, 1993

Gary P. Grotendorst, Ph.D.